

## Threonine 180 Is Required for G-protein-coupled Receptor Kinase 3- and $\beta$ -Arrestin 2-mediated Desensitization of the $\mu$ -Opioid Receptor in *Xenopus* Oocytes\*

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To determine the sites in the  $\mu$ -opioid receptor (MOR) critical for agonist-dependent desensitization, we constructed and coexpressed MORs lacking potential phosphorylation sites along with G-protein activated inwardly rectifying potassium channels composed of  $K_{ir}3.1$  and  $K_{ir}3.4$  subunits in *Xenopus* oocytes. Activation of MOR by the stable enkephalin analogue, [D-Ala<sup>2</sup>,MePhe<sup>4</sup>,Glyol<sup>5</sup>]enkephalin, led to homologous MOR desensitization in oocytes coexpressing both G-protein-coupled receptor kinase 3 (GRK3) and  $\beta$ -arrestin 2 (arr3). Coexpression with either GRK3 or arr3 individually did not significantly enhance desensitization of responses evoked by wild type MOR activation. Mutation of serine or threonine residues to alanines in the putative third cytoplasmic loop and truncation of the C-terminal tail did not block GRK/arr3-mediated desensitization of MOR. Instead, alanine substitution of a single threonine in the second cytoplasmic loop to produce MOR(T180A) was sufficient to block homologous desensitization. The insensitivity of MOR(T180A) might have resulted either from a block of arrestin activation or arrestin binding to MOR. To distinguish between these alternatives, we expressed a dominant positive arrestin, arr2(R169E), that desensitizes G protein-coupled receptors in an agonist-dependent but phosphorylation-independent manner. arr2(R169E) produced robust desensitization of MOR and MOR(T180A) in the absence of GRK3 coexpression. These results demonstrate that the T180A mutation probably blocks GRK3- and arr3-mediated desensitization of MOR by preventing a critical agonist-dependent receptor phosphorylation and suggest a novel GRK3 site of regulation not yet described for other G-protein-coupled receptors.

Opiates are the drugs of choice for the treatment of chronic pain, and a better understanding of the mechanisms underlying tolerance to opioids will undoubtedly lead to greater clinical utility. The molecular basis of tolerance manifests as a reduc-

tion in opioid agonist efficacy as demonstrated by a reduction in the rate of G-protein activation by the agonist-bound receptor complex (1–3). Furthermore, the modest reduction in cell surface receptor does not account for the observed decrease in agonist efficacy that accompanies tolerance measured biochemically (4, 5), cytochemically (6), or electrophysiologically (7). One mechanism of opioid receptor desensitization may be a receptor uncoupling from its effector system caused by receptor phosphorylation by a G-protein receptor kinase (GRK)<sup>1</sup> and subsequent binding of an arrestin.

The process of G-protein-coupled receptor (GPCR) desensitization can be resolved as a series of steps leading from GPCR activation to receptor uncoupling, internalization, and receptor recycling (Table I). This model has evolved from the studies done in a large number of laboratories but principally championed by the Lefkowitz group using the  $\beta$ -adrenergic receptor signaling as a prototypic GPCR (8, 9). In this scheme, GRKs phosphorylate the agonist-activated GPCR (8, 9). The phosphorylated GPCR induces a conformational change in arrestin, leading to arrestin activation (step 4), which unmasks arrestin's GPCR binding site and allows arrestin to bind the agonist-bound state of the GPCR (10–12). Arrestin binding then uncouples the GPCR from its effector by sterically blocking G-protein binding. Arrestin can also promote receptor internalization by serving as an adapter linking the GPCR-arrestin complex to dynamin and the clathrin-mediated endocytotic machinery (8, 9). The internalized GPCR-arrestin complex can subsequently be recycled to the plasma membrane in its pre-activated state following receptor dephosphorylation and disassembly of the complex. Alternatively, the arrestin-GPCR complex can be targeted to lysosomes for receptor degradation (8, 9).

We previously reported that homologous desensitization of MOR can be mediated by GRK and arrestin (13, 14). When MOR is coexpressed in the *Xenopus* oocyte heterologous gene expression system with G-protein-gated inwardly rectifying potassium channels  $K_{ir}3$  ( $K_{ir}3.1/3.4$ ), receptor activation by the selective MOR agonist, DAMGO, elicits a sustained increase in potassium conductance. Additional expression of both GRK3 and arr3 led to a dramatic increase in the desensitization rate of this MOR response (13, 14).

Strong evidence for a critical GRK phosphorylation site in

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<sup>1</sup> The abbreviations used are: GRK, G-protein receptor kinase; GRK3, G-protein receptor kinase 3 or  $\beta$ -ARK2; DAMGO, [D-Ala<sup>2</sup>,MePhe<sup>4</sup>,Glyol<sup>5</sup>]enkephalin; MOR,  $\mu$ -opioid receptor; arr2, arrestin 2; arr3, arrestin 3 ( $\beta$ -arrestin 2);  $K_{ir}3$ , G-protein-gated inwardly rectifying potassium channel (GIRK); GPCR, G-protein-coupled receptor; PCR, polymerase chain reaction; DOR,  $\delta$ -opioid receptor; KOR,  $\kappa$ -opioid receptor; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>.

TABLE I  
Lefkowitz model of GRK and arrestin regulation of  
G-protein-coupled receptors

Step	Action
1	Agonist activation of GPCR
2	GRK phosphorylation of agonist activated GPCR
3	Arrestin activation
4	Arrestin binding to agonist-activated and GRK-phosphorylated GPCR
5	Arrestin-mediated, clathrin-dependent endocytosis of GPCR
6	GPCR recycling or degradation

the C-terminal tail of MOR necessary for homologous MOR desensitization exists, although it remains a matter of controversy. Depending on both the expression system and the MOR agonist used, Thr<sup>394</sup> (15–18) or Thr<sup>354</sup>, Ser<sup>355</sup>, Ser<sup>356</sup>, and Thr<sup>357</sup> (19) or Ser<sup>356</sup> and Thr<sup>358</sup> (20), when substituted with alanines, have separately been shown to block MOR desensitization by GRKs and arrestins. Differences in the intrinsic GRKs and arrestins in the cell lines used may have caused the apparent discrepancies between the studies. In addition, the desensitization assays used did not clearly distinguish between a change in opioid tolerance caused by receptor uncoupling, internalization, and impaired receptor recycling. Our goal then was to dissect GRK- and arrestin-mediated regulation of MOR in a simpler system to more specifically define the critical GRK phosphorylation sites required for homologous MOR desensitization. To this end, we constructed MOR mutants lacking potential GRK phosphorylation sites and asked whether GRK3- and arrestin 3-dependent desensitization of MOR was affected.

#### EXPERIMENTAL PROCEDURES

**Chemicals**—DAMGO was from Peninsula Laboratories. Naloxone was from Research Biochemicals International. [<sup>3</sup>H]CTAP was from Multiple Peptide Systems. All other chemicals were from Sigma.

**Mutagenesis of MOR**—The rat MOR cDNA described previously (13, 14) was subcloned into the HindIII site of pBluescript (Stratagene), which was used for one of three PCR-based site-directed mutagenesis protocols described previously (21, 22). Depending on the protocol used, appropriate pairs of sense and antisense oligonucleotides and/or oligonucleotides designed to target the 5'- and 3'-ends of the MOR cDNA were used to generate the described deletions or substitutions of the MOR cDNA. The sense oligonucleotides for the site-directed mutagenesis were as follows: ttacgactcaagccgttcgcatgctagcgggcccacaagaa (S261A/S266A/S268A)  $\Delta$ 47 gatacgcaaaaatgaaggcggccgcaaca (T97A/T101A/T103A), and gccttgatttcgtgccccgcaaatgcaaaatcgttaacgtc (T180A). An adaptation of the QuickChange protocol from Stratagene was used to substitute serines 190, 195, and 195 to alanine. MOR(T97A/T101A/T103A) and MOR(T180A) cDNAs were made using the polymerase chain reaction overlap extension method (23). The resulting PCR products were subcloned into pGEM-T from Promega. All MOR cDNA templates for RNA synthesis were amplified from corresponding mutagenized clones using a 5' oligonucleotide (aatctagcatttagtgacactata-gaatagggccatggagacgac) that introduced an SP6 transcriptional recognition site and a 3' oligonucleotide (t<sub>30</sub>agggcaatggagcagttg) that introduced a 3' poly(A) tail. In the same manner, using the 5' oligonucleotide above and a 3' oligonucleotide (t<sub>30</sub>tcattggtgcagaaactctgaagca), we introduced a stop site corresponding to a 47-amino acid truncation of the translated MOR for the construction of MOR(S261A/S266A/S268A)  $\Delta$ 47 and MOR  $\Delta$ 47. All MOR mutations were confirmed by sequencing.

**Complementary DNA Clones and cRNA Synthesis**—All cDNA clones used in this study were described previously (14, 23). T7, T3, or SP6 mMESSAGE MACHINE kits (Ambion, Austin, TX) were used to generate capped cRNAs from the PCR templates of WT MOR and MOR mutants described under "Mutagenesis of MOR" or from linearized plasmid templates for rat GRK3 and bovine arr3.

**Oocyte Culture and Injection**—Defolliculated, stage IV oocytes were prepared as described (13). cRNA was injected (50 nl/oocyte) using a Drummond automatic microinjector, and then oocytes were incubated at 18 °C for 3–4 days in normal oocyte saline buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 5 mM HEPES, pH 7.5) solution supplemented with sodium pyruvate (2.5 mM) and gentamycin (50

μg/ml).

**Electrophysiology**—Oocytes were clamped at –80 mV with two electrodes filled with 3 M KCl having resistances of 0.5–1.5 ohms using a Geneclamp 500 amplifier and pCLAMP 6 software (Axon Instruments, Foster City, CA). Data were digitally recorded (Digidata 1200 (Axon Instruments) and an Intel 386 PC) and filtered. Membrane current traces were also recorded using a chart recorder. To facilitate the inward potassium current flow through the K<sub>ir</sub>3 channels, normal oocyte saline buffer (ND96) was modified to increase the KCl concentration to 16 mM, and the NaCl concentration was decreased correspondingly.

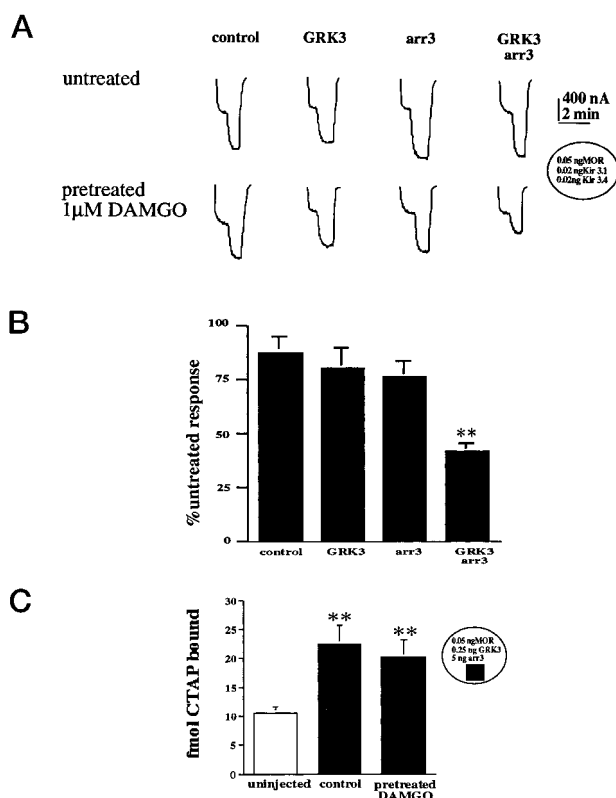
**Whole Oocyte [<sup>3</sup>H]CTAP Binding**—Oocytes were injected with 0.05 ng of MOR, 0.25 ng of GRK3, and 5 ng of arr3 cRNA and then 3 days later were either untreated or pretreated for 1 h with 1 μM DAMGO and washed three times with room temperature ND96. Each group was then incubated for 20 min in 20 nM [<sup>3</sup>H]CTAP (0.25 μCi/mmol) in ND96 at 4 °C. Four oocytes per group were placed on Whatman GF/C 25-mm circular glass microfiber filter paper under vacuum pressure and washed twice with 500 μl of cold ND96 and placed in 2.5-ml Ecolite scintillation fluid (ICN) for quantification of bound [<sup>3</sup>H]CTAP.

**Statistical Analysis**—Student's *t* test (with two-tailed *p* values) was used for comparison of the independent mean values. Dose-response curves were fitted to a simple Emax model using NFIT software (Island Products, Galveston, TX).

#### RESULTS

**GRK3- and arr3-mediated Desensitization of the MOR**—As described previously (13, 14), in oocytes injected with MOR, K<sub>ir</sub>3.1, and K<sub>ir</sub>3.4, DAMGO activation of MOR led to an increase in K<sub>ir</sub>3 current. Provided MOR expression was relatively low and both channel subunits, K<sub>ir</sub>3.1 and K<sub>ir</sub>3.4, were coexpressed, this MOR activation of K<sub>ir</sub>3 was remarkably stable, with only slight decreases in responsiveness during treatments as long as 12 h (14). This is in contrast to previous reports in which MOR activation of K<sub>ir</sub>3 currents in *Xenopus* oocytes, desensitized rapidly (up to 60% in 4 min) when MOR was expressed with K<sub>ir</sub>3.1 alone or when MOR was expressed at relatively high levels (24, 25). Under these latter conditions, the reduction in current observed was demonstrated to be heterologous desensitization, probably by receptor-independent channel inactivation (25). Thus, for this study the expression system was deliberately manipulated to minimize heterologous desensitization and to optimize the sensitivity of the system to homologous (GRK3- and arr3-mediated) desensitization of MOR. In addition, levels of MOR expression were adjusted to avoid the confounding effects of spare receptors.

As previously reported, coexpression of GRK3 and arr3 led to a marked decrease in MOR responsiveness after pretreatment with DAMGO (Fig. 1B) (13, 14). Peak MOR responses from oocytes injected with cRNA for MOR, K<sub>ir</sub>3.1, and K<sub>ir</sub>3.4, or also with GRK3 or arr3 cRNA under the two-electrode voltage clamp configuration were measured in oocytes clamped at –80 mV. Oocytes from each group were then incubated in 1 μM DAMGO for 30–60 min. Each oocyte was then washed for 10 min in normal oocyte saline buffer (ND96), and the peak MOR response to 1 μM DAMGO after agonist pretreatment was measured and compared with the response prior to DAMGO incubation. In oocytes injected with MOR and K<sub>ir</sub>3, responses after DAMGO treatment were greater than 75% of the pretreatment values, and expression of GRK3 or arr3 alone did not significantly alter the MOR desensitization (Fig. 1B). Representative traces of the MOR responses measured before and after DAMGO treatments are displayed in Fig. 1A. In contrast, coexpression of GRK3 and arr3 increased the extent of MOR desensitization (Fig. 1) as described previously (13, 14). The rate of desensitization was found to be most dependent on the levels of arr3 expression. For example, increasing the amount of cRNA injection for arr3 with the same levels of GRK3 cRNA injected led to MOR responses that desensitized to similar extents in less than 10 min (14). Conversely, decreasing the



**FIG. 1. GRK3- and arr3-mediated desensitization of MOR.** *A*, left to right, representative current traces from oocytes injected with 0.05 ng of MOR cRNA and 0.02 ng of each K<sub>ir</sub>3.1 and K<sub>ir</sub>3.4, also injected with 0.25 ng of GRK3, 5 ng of arr3, or both GRK3 and arr3. *A*, top, response to 1 μM DAMGO of above groups. Bottom, response to 1 μM DAMGO after a 30- or 60-min DAMGO incubation and 10-min wash in ND96. MOR responses in oocytes expressed with GRK3 and arr3 differed from batch to batch. The amount of time required for 50% of the WT MOR response to desensitize with GRK3 and arr3 expression was determined and then held constant for all experiments from the same donor on the same day. The time used in this series of experiments was usually between 30 and 60 min. Change in the oocyte perfusion medium from normal oocyte saline (ND96) containing 2 mM potassium to saline containing 16 mM potassium led to an increase in inward potassium current through the basally activated K<sub>ir</sub>. Activation of MOR by perfusion with 1 μM DAMGO caused a further increase in K<sub>ir</sub>3 current in oocyte saline buffer containing 16 mM potassium. The amount of MOR desensitization was calculated by comparing the peak DAMGO-elicited current before and after a 30–60-min incubation in 1 μM DAMGO and 10 min wash in ND96 and presented as the percentage of untreated response. Calibration for each trace was 400 nA, 2 min. *B*, summary of the WT MOR desensitization. DAMGO (1 μM) activation of MOR did not produce a significant amount of MOR desensitization in the absence of GRK3 and arr3 expression or in the presence of 0.25 ng of GRK3 or 5 ng of arr3 alone. Coexpression of GRK3 and arr3 dramatically increased the amount of MOR desensitization. Error bars, means ± S.E. for 9–16 independent determinations in oocytes from three oocyte donors. \*\*,  $p < 0.01$  compared with oocytes not injected with GRK3 and arr3. *C*, [<sup>3</sup>H]CTAP binding in either uninjected oocytes or oocytes injected with 0.05 ng of MOR, 0.02 ng of GRK3, and 5 ng of arr3 cRNA and then either untreated or pretreated for 1 h with 1 μM DAMGO. Following DAMGO treatment, oocytes were washed three times with room temperature ND96 to remove the opioid. In separate electrophysiological assays of opioid response, this wash procedure was sufficient to completely reverse the effects of 1 μM DAMGO (data not shown). Each group of intact oocytes was then incubated for 20 min in 20 nM [<sup>3</sup>H]CTAP (0.25 μCi/mmol) in ND96 at 4 °C, washed twice with 500 μl of cold ND96, and placed in 2.5 ml of scintillation fluid for quantification of bound [<sup>3</sup>H]CTAP. Each bar represents the average fmol of [<sup>3</sup>H]CTAP bound to four oocytes, from 7–10 separate determinations from two identical experiments. Error bars, means ± S.E.; \*\*,  $p < 0.01$  compared with uninjected oocytes.

arr3 cRNA injected required much longer DAMGO incubations for the same degree of MOR desensitization to occur (13).

To determine whether the reduction in apparent MOR re-

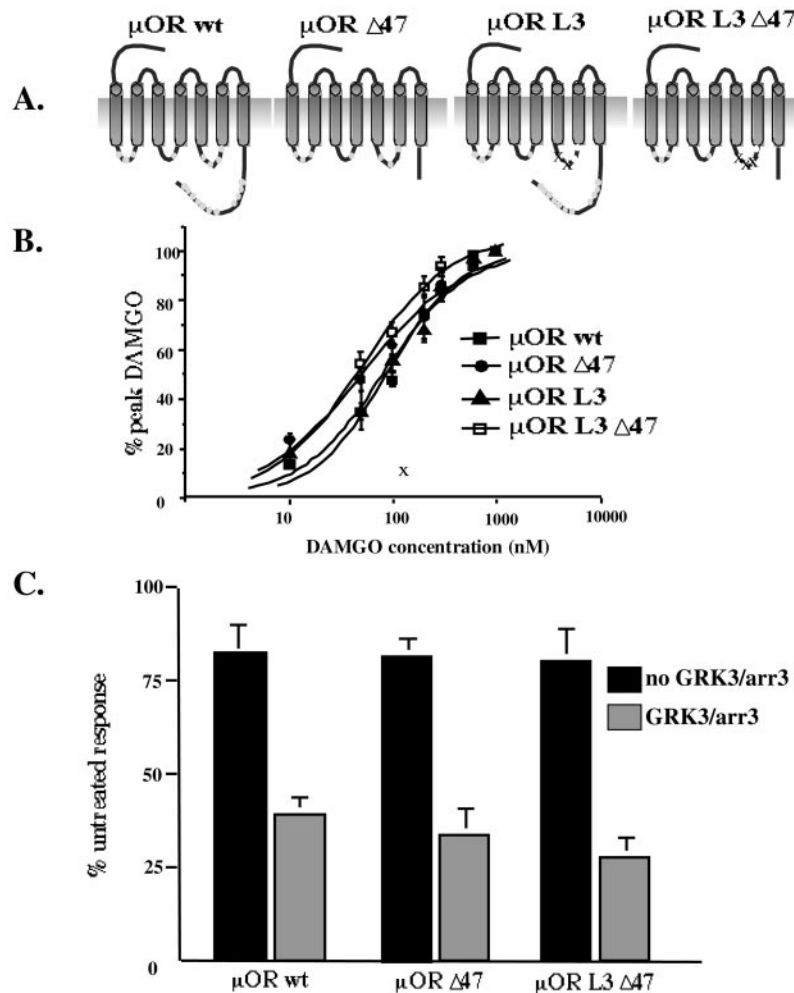
sponsiveness was caused by receptor internalization, we performed [<sup>3</sup>H]CTAP binding assays with whole oocytes under the same conditions in which MOR desensitization was measured electrophysiologically. [<sup>3</sup>H]CTAP is an antagonist; thus, it will not induce desensitization. Also, it is a charged peptide; thus, it will label only cell surface receptors (26). [<sup>3</sup>H]CTAP binding was significantly higher in oocytes expressing MOR than in uninjected oocytes (Fig. 1C). After treatment with 1 μM DAMGO for 60 min, specific binding of [<sup>3</sup>H]CTAP was not significantly changed in oocytes expressing MOR, GRK3, and arr3. This result demonstrates that the reduction in response seen electrophysiologically was not caused by receptor internalization.

**Dose-Response Relationships of WT MOR and MORs Lacking Potential GRK3 Phosphorylation Sites in the C-terminal Tail or the Third Cytoplasmic Loop**—To determine the critical phosphorylation sites important for the GRK3- and arr3-dependent desensitization described above, we began by removing the serines and threonines in the C-terminal tail by introducing a stop codon at residue 352 of MOR; MOR Δ47 lacks the last 47 amino acids (Fig. 2A). In addition, we substituted three of the serines in the third cytoplasmic loop of MOR (S261A/S266A/S268A) and introduced the C-terminal truncation resulting in MOR L3Δ47 (Fig. 2A). Cumulative dose-response curves to DAMGO for WT MOR, MOR L3, MOR Δ47, and MOR L3Δ47 were generated (Fig. 2B). EC<sub>50</sub> values for DAMGO activation of WT MOR, MOR Δ47, and MOR L3Δ47 were not significantly different. The result indicates that the binding affinity and intrinsic efficacy of MOR were not significantly altered by the respective alanine substitutions or truncations (Fig. 2B).

**GRK3- and arr3-mediated Desensitization of MORs Lacking Potential GRK3 Phosphorylation Sites in the Third Cytoplasmic Loop and C-terminal Tail**—GRK3 phosphorylation of the third cytoplasmic loop or the C-terminal tail has repeatedly been implicated in desensitization of G-protein-coupled receptors, and strong evidence exists for a critical role of the C-terminal tail in MOR desensitization (15–20). Thus, we coexpressed GRK3 and arr3 with WT MOR and MOR Δ47 to determine if the serines and threonines in the C-terminal tail were required for GRK3- and arr3-mediated desensitization of MOR in the *Xenopus* oocyte expression system. As with the WT MOR, MOR Δ47 did not desensitize significantly in the absence of GRK3 and arr3 expression. However, when coexpressed with GRK3 and arr3, MOR Δ47 desensitized at rate that was indistinguishable from WT MOR. The result demonstrates that the serine and threonine residues in the C-terminal tail were not necessary for GRK3- and arr3-mediated desensitization in this system. To determine whether either residues in the third cytoplasmic loop or the C-terminal tail were sufficient for GRK3- and arr3-mediated desensitization of MOR, we compared the desensitization of WT MOR and MOR L3Δ47, which lacked potential GRK3 phosphorylation sites in the third cytoplasmic loop and the C-terminal tail. As before, MOR L3Δ47 expression by itself did not lead to significant receptor desensitization. Coexpression of GRK3 and arr3 with MOR L3 Δ47, however, caused a MOR L3Δ47 desensitization that was indistinguishable from GRK3- and arr3-dependent desensitization of the wild type MOR. These data suggest that GRK3- and arr3-dependent desensitization of MOR in this system did not require phosphorylation of the third cytoplasmic loop or the C-terminal tail of MOR.

**Dose-Response Relationships of WT MOR and MORs Lacking Potential GRK3 Phosphorylation Sites in the First and Second Cytoplasmic Loop**—Since alanine substitution or removal of potential GRK3 phosphorylation sites of MOR in the third cytoplasmic loop and the C-terminal tail failed to block GRK3-



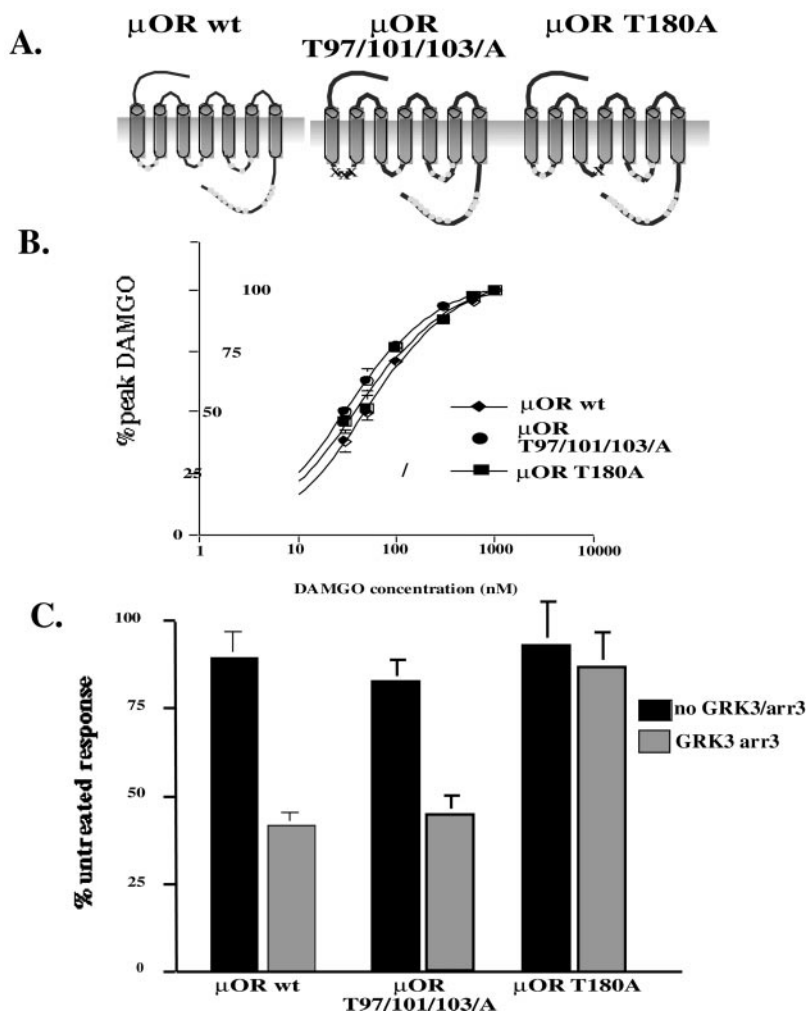


**FIG. 2. Effect of the third cytoplasmic loop and C-terminal tail mutations on DAMGO dose responses and GRK3- and arr3-mediated desensitization.** A, schematic diagrams depicting the WT MOR with serine and threonine residues represented as circles, and summarizing alanine substitutions (marked by X) and/or truncations made in the construction of MOR L3, MOR Δ47, and MOR L3Δ47. B, dose-response curves for DAMGO activation of MORs lacking potential phosphorylation sites in the third cytoplasmic loop and the C-terminal tail. Oocytes were clamped at  $-80$  mV while bathed in normal oocyte saline buffer containing 2 mM potassium. Oocytes were then superfused with a saline buffer in which the potassium concentration was increased to 16 mM, enabling a basal inward current to flow through the  $K_{ir}3$  channels. Cumulative, incrementing doses of DAMGO were applied to the bath followed by perfusion with the opioid receptor antagonist naloxone ( $1 \mu\text{M}$ ). The response to each dose of agonist was defined as the agonist-elicited inward current minus the basal current. The agonist response at each dose was normalized as a percentage of the average maximal DAMGO response obtained in separate oocytes from the same group. Each point represents the mean response measured in four to five oocytes. Oocytes were injected with 0.05–0.1 ng of the respective WT or mutant receptor cRNA along with 0.02 ng of cRNA of each  $K_{ir}3.1$  and  $K_{ir}3.4$ . Error bars, means  $\pm$  S.E.; when not shown, they are smaller than the plotted symbols. C, effect of third cytoplasmic loop and C-terminal tail mutations on GRK3- and arr3-mediated desensitization. Pretreatment with  $1 \mu\text{M}$  DAMGO did not produce a significant amount of MOR L3Δ47 or MOR Δ47 desensitization in the absence of GRK3 and arr3 expression or in the presence of 0.5 ng of GRK3 or 3 ng of arr3 alone. Coexpression of GRK3 and arr3 dramatically increased the rate of MOR desensitization that was not significantly different from WT MOR desensitization expressed with GRK3 and arrestin in the same batch and treated in the same way. Experimental design was identical to that described in the legend to Fig. 1. Error bars, means  $\pm$  S.E. for 5–10 independent determinations. \*\*,  $p < 0.01$  compared with oocytes not injected with GRK3 and arr3.

and arr3-mediated desensitization of MOR, we next constructed MOR(T97A/T101A/T103A) and MOR(T180A), which lacked potential GRK3 phosphorylation sites in the first and second cytoplasmic loop, respectively (Fig. 3A). To ensure that the described mutations did not alter receptor functioning, we constructed cumulative dose-response curves to DAMGO for WT MOR, MOR(T97A/T101A/T103A), and MOR(T180A) (Fig. 3B).  $\text{EC}_{50}$  values for DAMGO activation of WT MOR, MOR(T97A/T101A/T103A), and MOR(T180A) did not significantly differ, indicating that the receptor functioning of each receptor mutant was intact (Fig. 3B).

**GRK3- and arr3-mediated Desensitization of MORs Lacking Potential GRK3 Phosphorylation Site in the First and Second Cytoplasmic Loop**—To determine whether the critical phosphorylation sites of MOR necessary for GRK3- and arr3-dependent desensitization reside in the first or second cytoplasmic loop,

we coexpressed GRK3 and arr3 with MOR(T97A/T101A/T103A) and MOR(T180A) and compared the rates of desensitization with that of WT MOR. MOR(T97A/T101A/T103A) and MOR(T180A) did not desensitize significantly in the absence of GRK3 or arr3 expression (Fig. 3B). Furthermore, MOR(T97A/T101A/T103A), when expressed with GRK3 and arr3, desensitized at a rate that was indistinguishable from that of WT MOR under the same conditions (Fig. 4B). MOR(T180A), in which a single threonine was substituted for an alanine, however, failed to desensitize in the presence of GRK3 and arrestin coexpression. Instead, MOR(T180A), expressed with GRK3 and arr3, desensitized at rate that was indistinguishable from control rates in the absence of GRK3 and arr3 expression. As a positive control for GRK3 and arr3 expression in the oocytes injected with MOR(T180A),  $K_{ir}3$ , GRK3, and arr3, some of the oocytes from the following groups were also injected with cRNA



**FIG. 3. Effect of first and second cytoplasmic loop mutations on dose responses to DAMGO and GRK3- and arr3-mediated desensitization of MOR.** *A*, schematic diagrams depicting the WT MOR with serine and threonine residues represented as circles and summarizing alanine substitutions (marked by x) made in the construction of MOR(T97A/T101A/T103A) and MOR(T180A). *B*, dose-response curves for DAMGO activation of MORs lacking potential GRK phosphorylation sites in the first and second cytoplasmic loop. Conditions were the same as described for Fig. 2 except dose responses of MOR(T97A/T101A/T103A), MOR(T180A), and WT MOR were compared. Each point represents the mean response measured in four to five oocytes. Oocytes were injected with 0.05–0.5 ng of the respective WT or mutant receptor along with 0.02 ng of each  $K_{1/2}$  3.1 and  $K_{1/2}$  3.4. Error bars, means  $\pm$  S.E.; when not shown, they are smaller than the plotted symbols. *C*, summary of the WT MOR, MOR(T97A/T101A/T103A), and MOR(T180A) desensitization rates either expressed alone, with 0.1–0.5 ng of cRNA for each receptor injected, or expressed with 0.5 ng of GRK3 and 5 ng of arr3 or with 0.25 ng of GRK3 and 5 ng of arr3. Experimental conditions were identical to those described in the legend to Fig. 1. Desensitization of MOR responses with GRK3 and arr3 were not significantly different for MOR(T97A/T101A/T103A) compared with WT MOR expressed with GRK3 and arr3, whereas MOR(T180A) desensitization by GRK3 and arr3 was completely blocked under the same conditions on the same day. Error bars, means  $\pm$  S.E. for 5–12 independent determinations; \*\*,  $p < 0.01$  compared with respective receptor desensitization not injected with GRK3 and arr3.

for the  $\beta_2$ -adrenergic receptor and  $G_{\alpha_s}$ . As previously reported,  $\beta_2$ -adrenergic receptor activation by isoproterenol (1  $\mu$ M) activated Kir3, a response that desensitizes rapidly only in oocytes also coexpressing GRK3 and arr3.  $\beta_2$ -Adrenergic receptor desensitization rates in the oocytes injected with WT MOR; the WT MOR, GRK3, and arr3; and MOR(T180A), GRK3, and arr3 were  $4.6 \pm 1.9$ ,  $18.8 \pm 3.8$ , and  $16.7 \pm 1.7\%$  per min, respectively. The lack of significance between the latter two groups indicates that GRK3 and arr3 expressed well in oocytes expressing MOR WT and MOR(T180A).

In addition, the lack of GRK- and arrestin-dependent desensitization of MOR(T180A) was not due to receptor overexpression or a change in the intrinsic efficacy of MOR(T180A). Specific [ $^3$ H]CTAP binding to oocytes injected with 0.05 ng of cRNA for MOR WT or MOR(T180A) was not statistically different,  $6.7 \pm 0.9$  and  $4.7 \pm 0.7$  fmol bound, respectively ( $n = 10$ ). In addition, the DAMGO-evoked peak responses measured electrophysiologically were similar,  $620 \pm 157$  nA (MOR WT) and  $350 \pm 87$  nA (MOR(T180A)). Thus, the lack of GRK3/arr3-mediated

desensitization of MOR(T180A) did not result from a relative excess of MOR(T180A) expression compared with WT MOR expression. Furthermore, the average peak MOR response from oocytes injected with a higher dose of cRNA (0.1 ng) for each receptor was significantly higher for both receptor responses ( $1320 \pm 1220$  and  $1000 \pm 164$  nA for MOR WT and MOR(T180A), respectively); the result demonstrated a lack of a receptor reserve for each receptor at the dose of cRNA used. These data suggest that MOR(T180A) failed to desensitize because threonine 180 is required for GRK3- and arr3-dependent desensitization of MOR.

**Desensitization of WT MOR and MOR(T180A) by a Dominant Positive Arrestin, arr2(R169E)**—The insensitivity of MOR(T180A) presumably results from the loss of a critical GRK3 phosphorylation site. Alternatively, GRK3 could phosphorylate MOR(T180A) normally and activate arrestin, but the binding of the activated arr3 to MOR(T180A) might be impaired. To distinguish between these alternatives, we coexpressed WT MOR and MOR(T180A) with a form of arrestin

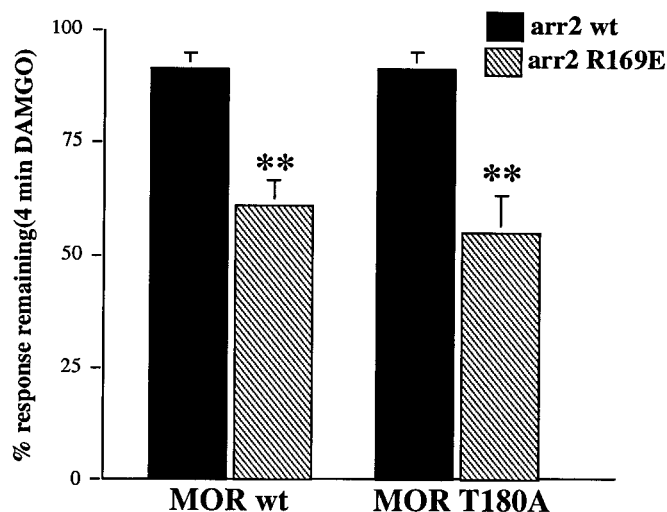


FIG. 4. **arr2(R169E)-mediated desensitization of WT MOR and MOR(T180A).** Summary of the WT MOR and MOR(T180A) desensitization in oocytes expressing 0.05 ng of WT MOR or MOR(T180A) with 16 ng of arr2 WT or 16 ng of arr2(R169E). arr2(R169E) expression with either WT MOR or MOR(T180A) caused a robust GRK-independent desensitization of both WT MOR and MOR(T180A) to extents that were statistically indistinguishable after 4 min of continuous perfusion with 1  $\mu$ M DAMGO. With the exception of the differences described above, experimental conditions were the same as described in Fig. 1. Error bars, means  $\pm$  S.E. for five independent determinations; \*\*,  $p < 0.01$  compared with respective receptor desensitization injected with WT arr2.

known to desensitize the  $\delta$  opioid receptor (DOR) and the  $\beta_2$ -adrenergic receptor in a manner that was agonist-dependent but GRK-independent (11). This "dominant positive" form of arrestin does not require activation by the phosphorylated GPCR, but it can bind and inactivate the agonist bound GPCR. Oocytes expressing arr2(R169E) showed robust agonist-dependent but GRK-independent, desensitization of MOR, whereas oocytes expressing WT arr2 did not show enhanced desensitization of MOR (Fig. 4). Furthermore, arr2(R169E) also caused robust desensitization of MOR(T180A) that was not statistically different from WT MOR desensitization by arr2(R169E). This enhanced desensitization of MOR by arr2(R169E) was not due to greater expression compared with arr2 WT. Expression of arr2 WT and arr2(R169E) in the oocytes used for in the desensitization experiments was found to be  $0.53 \pm 0.06$  and  $0.40 \pm 0.05$ , respectively, in the MOR WT-expressing oocytes and  $0.53 \pm 0.07$  and  $0.41 \pm 0.04$  in the MOR(T180A)-expressing oocytes (ng of arrestin/ $\mu$ g of protein  $\pm$  S.D.). These data suggest that the T180A mutation of MOR blocks GRK3- and arrestin-mediated desensitization, not by disrupting the arrestin binding to the receptor downstream of GRK action but because it removed a critical agonist-dependent GRK3 phosphorylation site necessary for arrestin activation.

#### DISCUSSION

The principal finding of the study was that threonine 180 of MOR was required for GRK3- and arr3-dependent homologous desensitization of MOR expressed in *Xenopus* oocytes. In our investigation, the removal of potential GRK phosphorylation sites in all other cytoplasmic domains of MOR failed to block MOR desensitization by GRK3 and arr3. Because receptor internalization does not contribute to the desensitization events in this system, the study clearly focuses on the roles of GRK3 and arr3 in the initial receptor uncoupling process. In addition, we further characterized the actions of the dominant positive form of arrestin. Previously, we demonstrated the agonist-dependent but GRK-independent desensitization of DOR and  $\beta_2$ -

adrenergic receptor by the dominant positive arr2(R169E). Here we report that arr2(R169E) also desensitized MOR in a GRK-independent but agonist-dependent manner. The observation that MOR(T180A) remained sensitive to arr2(R169E) suggested that MOR(T180A) lacked a critical GRK3 phosphorylation site necessary for homologous MOR desensitization.

From extensive studies of GRK and arrestin regulation of G-protein-coupled receptors, a common theme has evolved. Serine or threonine residues in the third cytoplasmic loop or the C-terminal tail have repeatedly been demonstrated to be responsible for the regulation of GPCRs by GRKs and arrestins. For example, GRK phosphorylation of muscarinic acetylcholine receptor  $m_1$  and  $m_2$  subtypes is predominately in the third cytoplasmic loop (27, 28). In contrast, the  $\delta$ - and  $\kappa$ -opioid receptors (DORs and KORs) require GRK phosphorylation of the C-terminal tail for GRK- and arrestin-dependent desensitization (13, 22). The difference between the critical site in MOR and the other GPCRs cannot be attributed to the differences in the expression system, since DOR and KOR desensitization were also characterized using the oocyte system.

Interestingly, the finding that homologous MOR desensitization in *Xenopus* oocytes does not require a C-terminal tail determinant was not the only disparity among these closely related opioid receptor subtypes in this system. Homologous MOR desensitization by GRK3 and arr3 proceeds with a dramatically slower time course compared with that of DOR and KOR. Although the rate of MOR desensitization can be accelerated with increased arrestin expression, under conditions where DOR and KOR desensitize in minutes, MOR desensitization required hours in this system (13, 22). The relatively slow desensitization rate of MOR might result from a slower kinetics of GRK3 phosphorylation, a less efficient activation of arrestin, or a slower association of activated arrestin with the GPCR. The observation that the dominant positive form of arrestin desensitizes MOR at rates that were equivalent to DOR and KOR desensitization rates suggests that the last explanation is unlikely. Our hypothesis is that GRK3-phosphorylated MOR is a less efficient activator of arrestin than either DOR or KOR, but this remains to be directly tested.

The findings that threonine 180 was required for the GRK- and arrestin-mediated desensitization of MOR and that the C-terminal tail was not involved are in sharp contrast with studies of this type in mammalian cell line expression systems (15–20). Although not agreeing on the exact residues responsible, prior studies of MOR desensitization in hypertransfected mammalian cell lines have pointed to sites within the C-terminal tail. The basis for the discrepancy between those studies and this one is not clear. The results using mammalian cell lines often rely on the intrinsic kinase and arrestins expressed; thus, the difference could be due to differences in GRK3 and arr3 and the unknown intrinsic proteins. The desensitization assays using mammalian cell lines are also strongly affected by internalization and receptor recycling rates, and overexpression of receptors produces a large opioid receptor reserve. The contributions of each of these to the tolerance observed would confound the measure of receptor desensitization. The receptor domains responsible for internalization and recycling are likely to be different from those responsible for receptor uncoupling. This distinction has been clearly demonstrated for cannabinoid and muscarinic acetylcholine receptors (21, 24). Furthermore, as discussed by Law *et al.* (30), recycling of MOR in mammalian cell lines can occur within minutes of agonist treatment such that the number of uncoupled receptors in cells highly overexpressing MOR may not be large enough to see a decrease in MOR-mediated second messenger responses. In addition, the presence of a large receptor reserve requires a



large fraction of receptor uncoupling before a significant change in the second messenger response can be measured. This is supported by those who have found a lack of correlation of MOR phosphorylation with receptor desensitization in cells highly overexpressing MOR (31, 32). This correlation was clearly demonstrated, however, when receptor recycling pathways were blocked or when the functional receptor number was decreased with the treatment of cells with an irreversible MOR antagonist (30). Since desensitization can potentially occur either by receptor uncoupling or internalization, to fully understand both processes it is necessary to have assays that distinguish these mechanisms and clearly define which is involved in terminating the MOR response in the system used.

For this reason, we deliberately expressed levels of MOR that were significantly less than those required to fully activate the coexpressed  $K_{ir}3$ . This ensured a lack of receptor reserve for the response measured and allowed us to measure receptor desensitization as it occurred. Furthermore, the finding that the C-terminal tail truncation that has been shown to block MOR internalization in mammalian cell lines did not affect MOR desensitization in this system suggests that MOR internalization in the manner that it occurs in mammalian cell lines is not responsible for the homologous MOR desensitization that we observed. This conclusion is supported by the finding that specific [ $^3$ H]CTAP binding was not decreased under conditions where MOR desensitization occurred. Therefore, it is likely that we have defined a critical role of Thr<sup>180</sup> of MOR necessary for receptor uncoupling that is distinct from the role of the C-terminal tail in MOR internalization.

Another intriguing possibility is that these data represent a homologue-specific action of GRK3 and arr3. In the *Xenopus* oocyte expression system, coexpression of GRK3 and arr3 was required for homologous MOR desensitization, which suggests the lack of endogenous enzymes that can substitute for either role. This is in contrast to mammalian cell expression systems where exogenous expression of GRK and arrestin is not required, making it difficult to clearly define roles of exogenously or endogenously expressed GRKs and arrestins.

Receptor uncoupling and internalization of MOR represent intimately related cellular processes that may be involved in the development of tolerance to opioid drugs. Thus, understanding these processes in greater detail may enable the elucidation of the roles of these processes separate from other mechanisms of opioid tolerance such as learning and memory and other compensatory changes in neuronal circuitry. Similarly, defining markers for receptor internalization distinct from receptor uncoupling may provide tools to elucidate the roles of each of these processes in opioid tolerance as well as

providing multiple targets for improving the clinical use of opioid drugs.

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